assumes no interaction between the solute and the macromolecule. However, since the sedimentation coefficients are usually very small (often between 1 and 2 S) – because of their large frictional ratios – and because of potential interactions with the separating medium, this method has not found major use for polysaccharide analysis or separation.

Polysaccharide Gels: Swelling Pressure

Sedimentation Equilibrium

If a gel is subjected to a centrifugal field, low enough to avoid sedimentation of the gel itself (typically $<$ 10 000 rpm), a concentration gradient will be established as in a conventional sedimentation equilibrium experiment on a solution. The gradient indicates the locally dependent de-swelling of the gel, which is caused by the swelling pressure generated by the centrifugal field. The concentration gradient will depend on the structure of the gel (number and strength of the cross-links) and whether the gel is reversible or not.

Sedimentation Velocity

At sufficiently high rotor speed ($>$ 10 000 rpm), the polymer concentration may drop to a sufficiently low level near the meniscus that a sol phase will appear: a conventional sedimentation velocity experiment can be performed, monitoring the movement of the boundary between gel and sol.

Data from sedimentation equilibrium and sedimentation velocity can be used to obtain the thermodynamic, elastic and structural parameters of the gel, complementing data from classical rheological approaches. Although gelatin has been the main focus of attention with this technique, several polysaccharide gels have been successfully characterized, such as carrageenan, pectin and alginate.

See also: **II / Particle Size Separation:** Theory and Instrumentation of Field Flow Fractionation. **III / Polysaccharides:** Liquid Chromatography.

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Liquid Chromatography

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Introduction

Separation, analysis and molecular weight distribution are very important for the characterization of polysaccharides in biochemistry, microbiology, agriculture and the food industry. During biosynthesis, a large range of components of different molecular weight are formed. Gas chromatography (GC) can be used for the analysis of very complex polysaccharide mixtures whereas high performance liquid chromatography (HPLC) is preferred for simple polysaccharide mixtures and for purification.

High Performance Liquid Chromatography

HPLC was introduced into the field of carbohydrate chemistry for the separation of mono- to tetrasaccharides of neutral sugars found in natural products

Table 1 Pre-column derivatization of carbohydrates for use in HPLC

Reagent	*Column*	*Mobile phase*
2-Aminoacridone	C18 reverse or normal-phase	Ammonium acetate–acetonitrile
2-Aminopyridine	C18 reverse or normal-phase	Acetonitrile–citrate buffer
Dansyl chloride	Spherisorb ODS2	Acetonitrile–water
Dansyl hydrazine	C18 reversed-phase	Acetonitrile–water
Phenylisocyanate	C18 reversed-phase	Acetonitrile–phosphate buffer
Benzoyl chloride	Silica	Ethyl acetate–hexane
o-Phthalaldehyde	C18 reversed-phase	Acetonitrile–methanol–water

of interest to the food industry. However, more recent applications have included the analysis and purification of oligo- and polysaccharides containing neutral and acetamido sugars and sialylated, sulphated and phosphorylated oligosaccharides.

Several different HPLC packing materials and solvent systems have been investigated for carbohydrate separation including native and derivatized oligosaccharides chromatographed on bare silica, bonded silica (NH_2-silica), silica modified with soluble amines *in situ*, reversed phase (RP), anion- and cation-exchangers and size-exclusion chromatography (**Table 1**). In several instances, a combination of different chromatographic modes is required for the complete separation of all isomers present in mixtures of polysaccharides obtained from biological sources.

Experimental Approach

There are a number of different HPLC processes for separating carbohydrates that depend on different chemical and physical properties for resolution. These analyses can be divided into ion-exchange processes at high temperature ($\geqslant 60^{\circ}C$) and adsorption (partition) processes at lower (room) temperatures. The most commonly used ion-exchange process involves the separation of borate complexes on quaternary ammonium anion-exchange resins (**Figure 1**). Borate forms complexes with most carbohydrates. The stability of these is influenced by the spatial arrangement of the alcohol groups involved, *cis* hydroxyl groups forming the most stable compounds. The columns may be run isocratically using borate buffers or by the use of gradient or stepwise elution using borate buffers of increasing molarity or containing increasing salt concentrations. The resolution obtained with this process normally increases with increasing temperature as long as the ion-exchange resin is stable.

Polysaccharides may be separated in all these processes using aqueous acetonitrile solvents. The variation of elution times of all the components with the separation parameters (e.g. temperature, acetonitrile concentration, buffer pH) must be determined by computer analysis or by visual inspection of the curves. Microbial growth inhibitor should be present in all aqueous phases.

Techniques for Carbohydrate Chromatography

By using different approaches to synthesis, stationary phases for almost any known type of chromatographic interaction can be provided. Continuous porous polymer rods can, for example, be produced *in situ* by thermal or photopolymerization on suitable monomers. These stationary phases can be transformed to adapt them for ion exchange or hydrophobic interaction chromatography. A more specific interaction can be introduced by 'imprinting' the polymers during synthesis with the future analyte (enantioselective/affinity stationary phases). In a different approach, a sol-gel technique can be used to produce stationary phases on a silica basis. These phases are mechanically more stable and were, for instance, used to separate polyaromatic carbohydrates and sugars within minutes.

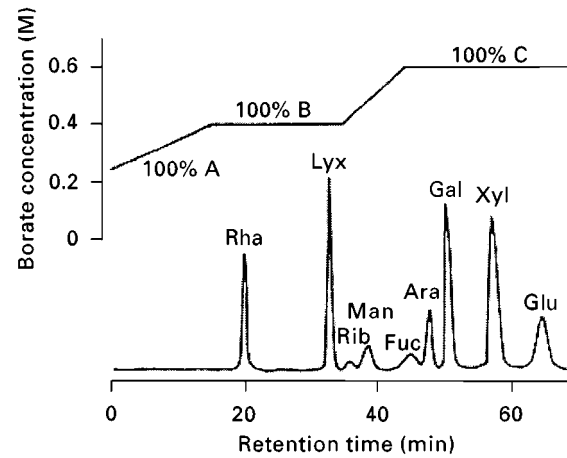

Figure 1 Analysis of an equimolar mixture of aldoses. A mixture of 15–60 nmol of L-rhamnose (Rha), D-lyxose (Lyx), D-ribose (Rib) D-mannose (Man), L-fucose (Fuc), L-arabinose (Ara), D-galactose (Gal), D-xylose (Xyl) and D-glucose (Glu) was dissolved in 100 μL of water and applied to a Hitachi No. 2633 resin column (8 mm i.d. × 8 cm). The packing is a quaternary ammonium resin with an average bead diameter of 11 μm. The borate gradient (1 mL min^{-1}) was used to elute the carbohydrates using buffers A (0.25 mol L^{-1}, pH 8.2), B (0.4 mol L^{-1}, pH 7.4) and C (0.6 mol L^{-1}, pH 9.3). Post-column detection was by fluorimetry after reaction with 2-cyanoacetamide–borate. (Reproduced with permission from Chaplin MF and Kennedy JF (eds) (1994) *Carbohydrate Analysis: A Practical Approach*, 2nd edn, p. 17. Copyright IRL Press Limited.)

Adsorption chromatography Adsorption, or normal phase, chromatography relies on the surface hydroxyl groups of silica (and to a lesser extent alumina) which can interact with solutes and effect a separation on account of the different strengths of interaction. The separation of neutral oligosaccharides cannot be carried out conveniently by this method although limited separations can be achieved in water or by using aqueous–organic mixtures. The method is well suited to the analysis of derivatives of oligosaccharides of low degree of polymerization using non-aqueous eluents. The use of high concentrations of organic solvents in an aqueous eluent gives rise to problems of solubility of polysaccharides.

Bonded-phase chromatography By far the most frequently used systems for separation of oligosaccharides are those using chemically bonded phases that fractionate materials on the basis of their relative affinities for the mobile phase and the bonded phase. The two most important types of column are those containing the aminopropyl-bonded phase and a hybrid phase containing cyanopropyl- and aminopropylsiloxane-bonded ligands.

Separation of series of oligosaccharides from, for example, hydrolysed starch can readily be achieved with up to a degree of polymerization of 10 in 15–20 min using acetonitrile–water eluents containing 35–40% water (**Figure 2**). Increasing the water content to 45% can increase the number of detectable oligosaccharides up to about 15 units. However very high-molecular-weight materials cannot be analysed due to excesive retention and solubility problems in acetonitrile–water eluents. For a full analysis, separation by gel permeation or ion exchange is also required.

Instead of using a bonded silica aminopropylsiloxane-bonded phase, an alkyldiamine or polyalkylamine can be added to the eluent forming a dynamic equilibrium between an amine-containing phase coating the silica and that in the eluent. Separations are similar to those obtained using chemically bonded aminopropyl sorbents with separations of up to 20–25 units in 45 min with eluents containing 50% water in acetonitrile (**Figure 3**).

Ion-exchange chromatography Anion-exchange resins of styrene-divinylbenzene matrices in the sulfate form have been reported for the separation of the simple disaccharides using eluents of 80–90% ethanol in water. Rapid and efficient separation of simple and complex carbohydrates by high performance anion-exchange chromatography is obtained using a quarternized alkylamine sorbent made of

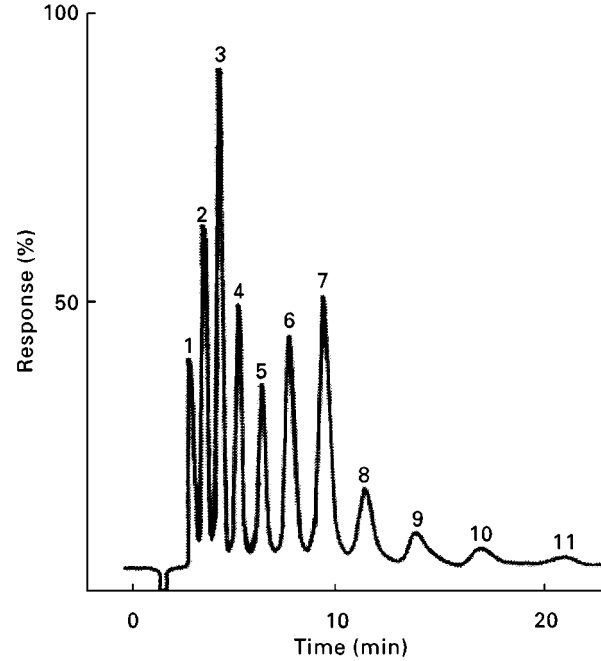

Figure 2 Separation of starch-derived oligosaccharides on a Spherisorb S5 NH_2 column (1, 2, 3, etc., refer to the degree of polymerization of the oligosaccharide). (Reproduced with permission from Chaplin MF and Kennedy JF (eds) (1994) *Carbohydrate Analysis: A Practical Approach*, 2nd edn, p. 48. Copyright IRL Press Limited.)

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chemically modified nonporous and monodisperse highly cross-linked styrene copolymer particles having a diameter of 2.5 μm and a surface area of 3 $m^2\ g^{-1}$. In this technique there are many advantages of using short columns instead of those currently used in high performance anion-exchange chromatography in terms of separation time.

On the other hand, cation resins in the lithium form have been used for similar analyses using 90% ethanol in water eluents. The use of 4 and 8% cross-linked cation-exchange resins in the calcium or silver form have been used to provide a rapid separation of oligosaccharides. It is possible to obtain a total analysis of material applied to the column and the use of water as the only eluent (**Figure 4**).

An early approach to reduce stationary phase mass transfer resistance was to form thin ion-exchange shells on the surface of an impervious core, e.g. glass beads to give 'pellicular' materials. The major application of pellicular anion exchangers is in the chromatography of carbohydrates at >pH 12, where most carbohydrates become anionic and can be separated on a column packed with a strong anion exchanger. Because carbohydrates undergo chemical changes on prolonged exposure to strong alkali, the separation time must be short.

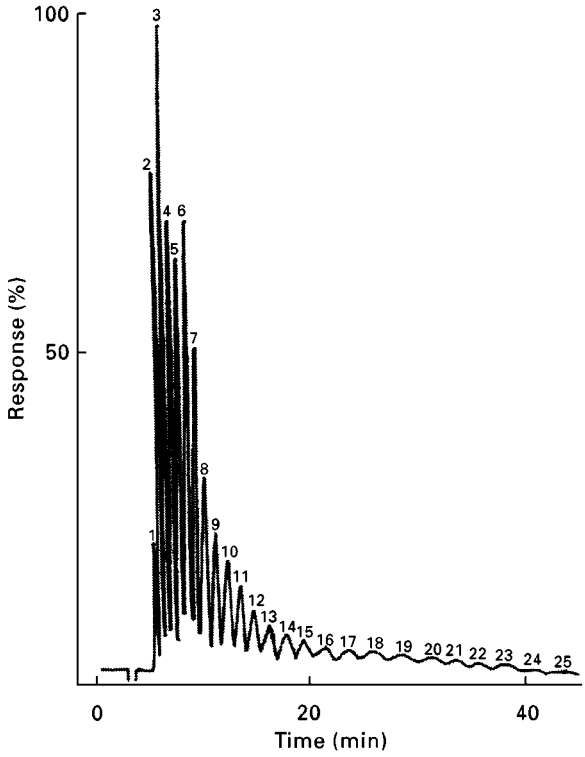

Figure 3 Separation of starch-derived oligosaccharides on an *in situ*-modified silica column using 1,4-diaminobutane (0.01% v/v) as modifier. Numbers refer to the degree of polymerization of the oligosaccharide. (Reproduced with permission from Chaplin MF and Kennedy JF (eds) (1994) *Carbohydrate Analysis: A Practical Approach*, 2nd edn, p. 49. Copyright IRL Press Limited.)

Gel permeation chromatography There has been no direct replacement for the cross-linked polysaccharide or polyacrylamide materials used for traditional gel permeation analysis of oligosaccharides. Some advances have been made with the development of silica matrices deactivated by chemical bonding of an organic ether stationary phase to provide a hydrophilic surface.

Fractionation ranges extended down to 10–12 units whilst bare silica can extend the fractionation range down to 5–6 units. Even with the modified materials, adsorption effects are present and elution with ionic buffers is recommended within the pH range 2–7.

Water-compatible hydroxylated polyether-based matrices have been developed which overcome some of the disadvantages of silica-based materials and have fractionation ranges which are comparable to the cross-linked polysaccharide and polyacrylamide gels. In spite of their lower selectivity compared to silica-based matrices, such materials have a very high stability towards alkaline pH (up to pH 12). Separation times are of the order of one-third to one-tenth that of traditional low-pressure gel permeation analysis, but they are inferior to those currently obtained by ion-exchange chromatography. Consequently, less emphasis is placed on high performance gel permeation chromatography for oligosaccharide fractionation.

Detection Systems

Detection of oligosaccharides eluting from HPLC columns is the biggest challenge and weakest link in the analysis of polysaccharides. When sensitivity in the submicrogram range is required the only method readily available is the use of pre-column derivatization with separation via adsorption chromatography and detection via UV monitoring or pulsed electrochemical detection at noble metal electrodes. Attempts to use strong cation-exchange resins to hydrolyse the glycosidic bonds in polysaccharides to give complete conversion to monosaccharides and lower oligosaccharides after chromatographic separation has been reported with the resulting saccharides being detected as reducing compounds.

Direct Detection

UV absorbance Carbohydrates do not absorb light in the UV or visible range and have no fluorescence. Polysaccharides, however, do absorb at wavelengths in the far UV. The higher absorbance is at about 188 nm but, due to noise in the detection signal below 190 nm, detection is normally performed at wavelengths between 192 and 200 nm. The response, depending largely on the freedom of the carbonyl

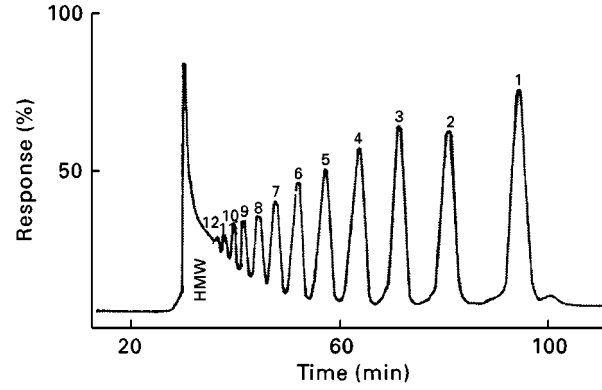

Figure 4 Separation of starch-derived oligosaccharides by high performance ion-exchange chromatography using 4% cross-linked cation-exchange resin with silver counterions (1, 2, 3, etc., refer to the degree of polymerization of the oligosaccharide; HMW, high-molecular-weight material above). (Reproduced with permission from Chaplin MF and Kennedy JF (eds) (1994) *Carbohydrate Analysis: A Practical Approach*, 2nd edn, p. 50. Copyright IRL Press Limited.)

group, differs between the polysaccharides. Analyses are restricted to solvents such as acetonitrile–water mixtures that do not absorb significantly at these wavelengths. Oligosaccharides containing acetamido groups or sialic acids can be detected by absorbance in the range 180–220 nm with a detection limit of approximately 1 nmol. The best signal-to-noise ratio for a range of carbohydrates is found between 195 and 210 nm.

For preparative separations, a higher wavelength can be used. The sensitivity decreases by 10- and 100-fold by increasing the wavelength of detection from 208 to 218 and 228 nm. Polysaccharides having unsaturated monosaccharides formed by enzymic digestion of sulfated glycosaminoglycans have an absorption maximum at 232 nm. The sensitivity of detection of these oligosaccharides at 232 nm is less than 1 nmol.

The sensitivity can be further improved (down to 1 pmol) by introducing a strongly UV-absorbing group such as benzoyl or phenylisocyanate into the molecules. Diode array detection is a technique widely used in combination with reversed-phase HPLC for the separation and quantitative determination of polysaccharides. The detection limit obtained by this method is about 5 pmol. In the determination of the degree of polymerization of agar-type polysaccharides, samples are prepared adding 1 g of starch to 10 mL of 0.2 mol L^{-1} trifluoroacetic acid, heating at 100°C for one hour, then adding 90 mL of absolute ethanol and storing at −70°C to allow precipitated saccharides to settle. It is also necessary to remove all remaining traces of ethanol by evaporation under nitrogen since ethanol interferes with retention of the larger oligomers. Finally, the dried residue is redissolved and filtered in deionized water prior to injection.

The simplest and ideal methods of detection are those that do not require chemical derivatization of the sugars but in order to increase the sensitivity of detection, they may be derivatized to give light-absorbing or fluorescent compounds before or after LC separation. This approach may be very useful, especially if it allows the use of efficient chemically bonded or underivatized silica columns.

The sensitivity of carbohydrate detection can be greatly increased by introducing a radioactive label, either by reduction with sodium boro[^{3}H]hydride or de-*N*-acetylation and re-*N*-acetylation with [^{14}C]acetic anhydride. Oligosaccharides isolated from biological sources are often obtained in reduced and/or de-*N*-acetylated form. Analysis of reduced oligosaccharides has an additional advantage in that reduction destroys the anomerization at the reducing end, thus simplifying chromatography and subsequent structural analysis.

The separation of anomers is also given by HPLC after pre-column derivatization of reducing oligosaccharides with acetyl, benzoyl and phenylisocyanate groups. Therefore, reduction is usually performed routinely as part of these derivatization procedures. The sensitivity of detection of the latter two derivatives by UV would usually obviate the need to introduce a radioactive label.

Several online radioactive detection systems are available with both solid scintillant cells and the possibility of addition of liquid scintillant, for example, from Berthold, Beckman and Nuclear Enterprises. For ^{3}H-labelled oligosaccharides, approximately 10^4 and 10^2 cpm are required for detection by the two types of measuring technique. The sensitivity of detection of ^{14}C is of order of 10^3 cpm.

Early post-column derivatization methods for carbohydrate detection employed strong acids and thus required acid-resistant equipment. For example, the use of orcinol–concentrated sulfuric acid reagent required an elaborate carbohydrate analyser to be set up for direct post-column detection. Similar methods, such as the phenol–sulfuric acid assay, have proved useful for separate, off-column hexose determination. The sensitivity of detection for these methods is approximately 20 nmol hexose which is slightly less than by UV detection of native oligosaccharides containing hexose and hexosacetamido sugars, but represents a greatly improved sensitivity for detection of oligosaccharides containing neutral sugars alone.

More recent post-column derivatization methods have used noncorrosive reagents, particularly for detection of reducing sugars after borate-complex ion-exchange chromatography. The copper complexes of 2,2′-bicinchoninate, ethanolamine–boric acid and 2-cyanoacetamide, for example, have been used to detect 1 nmol oligosaccharide. Detection of less than 1 nmol oligosaccharide has been reported using tetrazolium blue reagent (3,3′-[3,3′-dimethoxy-1,1′-biphenyl-4,4′-diyl]bis[2,5-diphenyl-2H-tetrazolium] dichloride) which has the additional advantage of achieving this sensitivity in the absence of borate buffer and at a lower reaction temperature of 85°C. The method is suitable for both reduced and reducing oligosaccharides.

The ammoniacal cupric sulphate assay is sensitive but simple and avoids the corrosive reagents and complex heating/mixing protocols of some other post-column detection methods. It is of general application to substances that react with cuprammonium, for example, carbohydrate derivatives and glycols, and is not sensitive to changes in the solvent composition. Monosaccharides do not seriously interfere in this assay if the periodate oxidation reaction

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takes place below 40°C and they are not present in an overwhelming excess. They can be detected if the periodate oxidation is allowed to take place at a higher temperature (e.g. 100°C). Periodate oxidation alone may be used for post-column detection of cyclitols, aldoses, alditols and ketoses by monitoring the absorbance at 260 nm. Assay with cyanoacetamide uses noncorrosive reagents, shows good linearity and is highly sensitive for aldoses, hexosamines and alduronic acids. The fluorescence is quenched by acetonitrile, if present in the eluate, but the absorbance is unaffected.

Refractive index and light-scattering detection A detection method of general applicability makes use of changes in refractivity. It is at least ten times less sensitive than UV detection due to high background noise caused by temperature fluctuations an pump pulsations.

Many of the early HPLC studies on carbohydrates used a differential refractive index detector because these studies were carried out on oligosaccharides containing neutral sugars only, which absorb weakly in the UV region. The disadvantages of using a refractometer are that only isocratic elution is possible and the sensitivity is relatively low, 10–100 nmol being required for detection.

Light scattering (LS) can be used for measuring the molar mass of polysaccharides. Several polysaccharides have been routinely characterized by LS for the determination of the molecular weight distribution. The molecular weight distribution can be used to determine the polydispersity (M_w/M_n) and the heterogeneity in a sample.

Electrochemical detection of polysaccharides Nanomolar detection of both reducing and reduced neutral oligosaccharides and polysaccharide hydrolysates has been reported using pulsed amperometric detection (PAD) employing a gold electrode. This method is stated to have the advantage of increased detector durability compared with potentiometric and single potential detectors. Detection of carbohydrates in the presence of a high concentration of potentially interfering salts is also possible. An increased sensitivity for detection of reducing sugars (down to 1 pmol) has been reported using amperometric detection of polysaccharides chromatographed in sodium phosphate buffer after post-column reaction with copper bis(phenanthroline) in alkaline solution at 96°C.

PAD, in conjunction with high-pH anion-exchange chromatography, has also become the method of choice for analysing sugars. Their direct electrochemistry on noble metal electrodes suffers from electrode fouling due to absorption of oxidized species on the electrode surface. PAD overcomes this problem by using a triple-step potential waveform. In the first step (data recording), the gold electrode is held at a potential suitable for oxidation of the analyte. The second step raises the potential to some higher value, where absorbed oxidation products are oxidized further into mobile phase-soluble products, thereby cleaning the electrode. Finally, the electrode generates a new gold oxide surface. Using this technique, closely related saccharides can be separated and detected in the 10 to 100 pmol range.

Analysis of Complex Mixtures

HPLC of glycopeptides is not widely developed because of peptide interferences. In fact, a given glycan located in a given peptide sequence of the protein generally gives rise to a mixture of glycopeptides due to the random nature of proteolytic action. Chemical or enzymatic removal of glycans from such complex mixtures solves this problem and allows the use of HPLC. It is possible to use different HPLC techniques such as:

- anion-exchange chromatography of sialyloligosaccharides (**Figure 5**A);
- partition chromatography of neutral and acidic oligosaccharides on primary amine-bonded silica or alkyl diol-bonded silica (**Figure 5**B);
- reversed-phase chromatography of neutral oligosaccharides on C_2 and C_{18}-bonded silica (**Figure 5**C).

Conjugation of two fluorescent ortho-substituted aniline derivatives, 2-aminobenzamide (2-AB) and 2-anthranilic acid (2-AA), to *N*- and *O*-glycans have been recently investigated. Conjugation conditions for attaching 2-AB and 2-AA to core-fucosylated and nonfucosylated glycans have been developed using complex *N*-glycans radiolabelled at the nonreducing terminus with $[^3H]C_6$-galactose.

Most glycolipid separations have involved derivatizing the glycolipids to allow UV detection. The derivatives are usually benzoyl or *p*-nitrobenzoyl esters and the columns are based on silica gel. For instance, ceramides (0.1–1.0 mg) are dissolved in 20% benzoyl chloride in 0.6 mL of dry pyridine and heated at 60°C for 1 h. The solvent is evaporated to dryness in a stream of N_2 and taken up in a small volume of hexane for injection into the liquid chromatograph. The mobile phase is a linear gradient system of 0.20% methanol to 0.75% methanol in hexane. The detector operates at 254 nm and the minimum detection is about 10 pmol of each glycolipid.

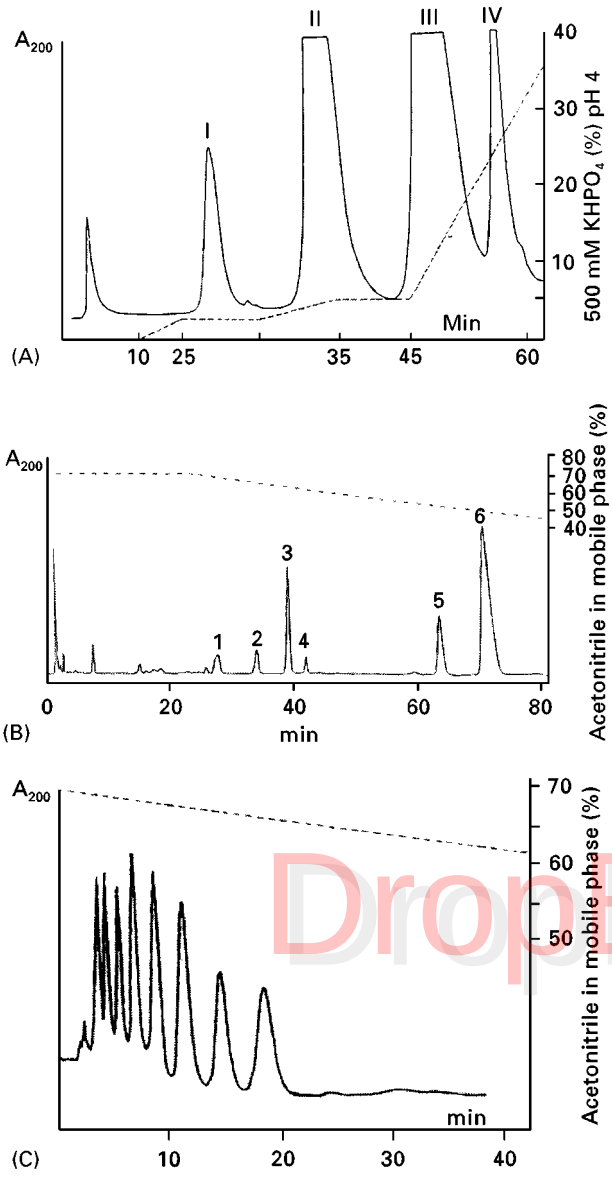

Figure 5 (A) HPLC on 10 μm Micropak AX-10 column of sialoglycans liberated by hydrazinolysis of α_1-acid glycoprotein. I, II, III, IV: mono-, di-, tri- and tetrasialylated glycans. The recovery was 91%. (B) HPLC on 5 μm of glycan-alditols liberated by β-elimination from Cad erythrocyte membrane glycophorin. 1: NeuAc(α2–3)Gal(β1–3)GalNAc-ol; 3: NeuAc(α2–3)GalNAc(β1–4)Gal(β1–3)GalNAc-ol; 5: NeuAc(α2–3)Gal(β1–3)[NeuAc (α2–6)]GalNAc-ol; 6: NeuAc(α2–3)[GalNAc(β1–4)]Gal(β1–3) [NeuAc(α2–6)]GalNAc-ol. (C) HPLC on alkyl diol-bonded silica of oligomannoside-alditols from the urine of a patient with a mannosidosis. M_2G-ol to M_9G-ol: oligomannoside-alditols containing from 2 to 9 mannose residues. (Reproduced with permission from Chaplin MF and Kennedy JF (eds) (1994) *Carbohydrate Analysis: A Practical Approach*, 2nd edn, pp. 155, 157 and 158. Copyright IRL Press Limited.)

The *p*-nitrobenzoyl derivatives are more sensitive to UV detection than the benzoyl derivatives but the benzoyl derivatives are better separated than the *p*-nitrobenzoyl derivatives. A problem exists for regenerating the native glycolipide. It is not too difficult to remove *O*-benzoyl groups but the *N*-benzoyl group on amino sugars and in the ceramide fragment are far more difficult to remove. The use of a catalyst such as 4-dimethylaminopyridine in the benzoylation reaction with benzoic anhydride produces only *O*-benzoyl substitution.

Determination of the Position of Glycosidic Linkages. Combined HPLC and Mass Spectrometry (HPLC–MS)

Oligosaccharides released from human transferrin have been derivatized with 2-aminoacridone (2-AMAC) prior to analysis by either reversed- or normal-phase HPLC. Collected fractions of 2-AMAC-derivatized glycans have been analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, before and after desialylation (**Figure 6**).

A procedure for analysis of a mixture of neutral and acidic sugars in bacterial whole-cell hydrolysates using high performance anion-exchange liquid chromatography–electrospray ionization–tandem mass spectrometry (HPAEC–ESI–MS–MS) has been described. HPAEC is well established as a high-resolution chromatographic technique, in conjunction with pulsed amperometric detection. Alternatively, for more selective detection, sugars (as $M-H^-$ ions) are monitored using ESI–MS. Sugar identification is achieved by MS–MS using ESI.

Another simple, sensitive method for the structural characterization of oligosaccharides by fast atom bombardment–mass spectrometry (FAB–MS) has been designed. Oligosaccharides are labelled with a UV chromophore (which also serves as a charge-stabilizing group) and with a hydrophobic alkyl tail. The chromophore, a 2,4-dinitrophenyl group, aids UV detection and stabilizes negative ion species formed during analysis by FAB–MS. The hydrophobic tail, provided by an octyl group, enhances the surface activity of the analytes and makes them amenable to separation on a C18-bonded phase. This method has been applied to the structural analysis of the components of a mixture of starch maltodextrins with a degree of polymerization 1–16, to the analysis of the structure of pure maltohexose, and to a previously characterized oligosaccharide from *Rhizobium* capsular polysaccharide.

HPLC technology has been developed that is capable of resolving subpicomolar quantities of mixtures of fluorescent-labelled neutral and acidic

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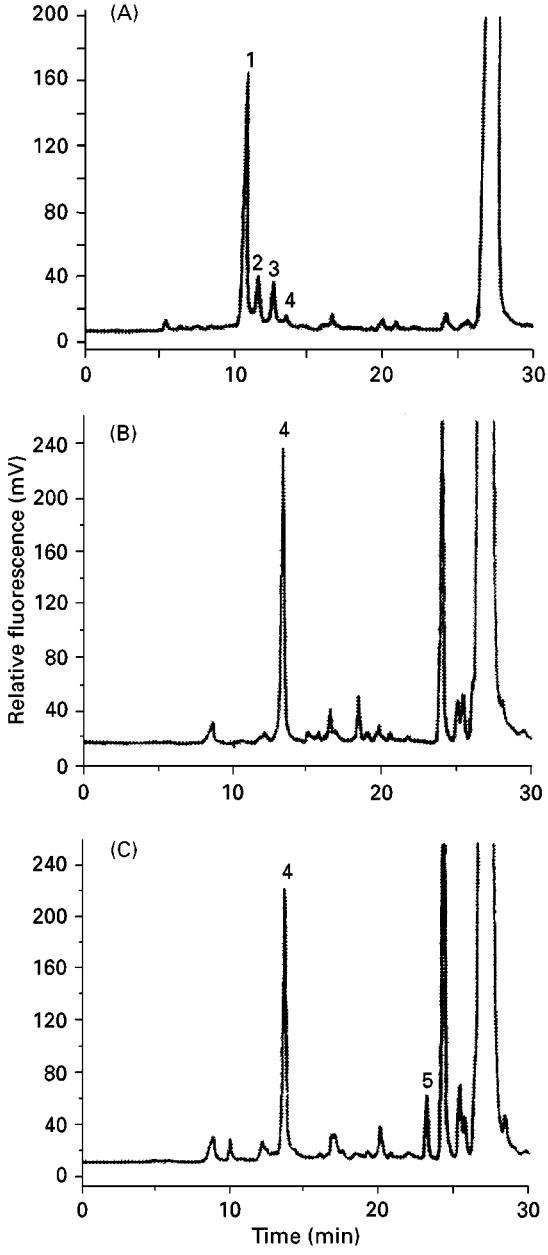

Figure 6 Reversed-phase HPLC analysis of glycan mixture from transferrin: (A) untreated; (B and C) digested with sialidase and a mixture of sialidase and fucosidase, respectively, prior to derivatization with 2-aminoacridone. (Reproduced with permission from Charlwood J, Birrell H, Tolson D and Camilleri P (1998) *Analytical Chemistry* 70: 2531. Copyright American Chemical Society.)

glycans simultaneously and in their correct molar proportions. The reproducibility of the separation system, the predictability of glucose unit values, and the quantitative response of the detection system for individual fluorescently labelled glycans allows automation for the analysis of neutral sugars using a combination of enzymes as in the reagent array analysis method (RAAM). In addition, the simultaneous resolution of both acidic (sialylated) and neutral products from the RAAM digestion allowed direct analysis of sialylated glycans, eliminating the previous need to remove sialic acid residues in a preliminary step.

Future Developments

As shown above, the greatest advances in the detection and characterization of polysaccharides will evolve from advances in mass spectrometry. The current ability to identify, with little ambiguity, virtually any high-molecular-weight biopolymer via the electrospray interface between HPLC and MS will continue to improve, in terms of ease of use, lower sample requirements and higher molecular-weight ranges. Advances will use detection techniques incorporating the strengths of each technique in sequential detection schemes. UV detection prior to MS is already common. Improvements will also come about with regard to the mapping of carbohydrates and polysaccharides.

The emphasis for the future does not appear to reside in reaction detection for HPLC of polysaccharides, but rather in more sophisticated instrumental methods of detection. Electrochemistry seems to hold much appeal, and yet it has not realized its full potential. Pulsed amperometric detection methods are maturing: chemically modified electrodes can be much more selective and sensitive than glassy carbon or Au/Hg-type electrodes. Multiple-array detectors, perhaps with chemically modified electrodes or different noble metal electrodes (Ni, CuO), may also provide more information of a qualitative nature. Current detection methods such as UV–visible, fluorescence and light scattering will always be useful for qualitative information, identification of the chromatographic performance of a peak, and for absolute quantitation, but they may never provide 100% specific information about the structure. On the other hand, the use of circular dichroism or optical rotary dispersion for detection of the conformation of polysaccharides can be equally used.

See also: **II/Chromatography: Liquid:** Derivatization; Detectors: Fluorescence Detection; Detectors: Ultraviolet and Visible Detection; Mechanisms: Ion Chromatography; Mechanisms: Size Exclusion Chromatography. **III/Carbohydrates:** Gas Chromatography and Gas Chromatography-Mass Spectrometry; Liquid Chromatography. **Ion-Exclusion Chromatography: Liquid Chromatography.**

Further Reading

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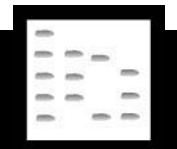
POROUS GRAPHITIC CARBON: LIQUID CHROMATOGRAPHY



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Introduction

Various carbonaceous sorbents have been used successfully since the early days of gas chromatography (GC), but for many years their application to liquid chromatography (LC) was unsuccessful. Active carbons with high specific surface areas were shown to be microporous and to contain polar groups at their surface, which provided poor LC performance. Graphitized carbon blacks (GCBs) lacked sufficient mechanical strength to withstand high LC pressures, in addition to having polar surface groups. In the 1970s, bonded silicas were extensively developed, but they had some disadvantages including solubility in the eluents, hydrolysis of the bonded chain at low or high pH, and the effects of the unavoidable unreacted silanol groups. Several attempts were made to prepare graphite-based sorbents that would not suffer from the disadvantages of bonded silica sorbents, but it was not until 1979 that Knox and Gilbert patented a method for making a robust porous carbon that possessed the required properties for use in LC. An improved version of this material became commercially available in 1988 under the tradename Hypercarb®. Although one or two other carbons made by Japanese workers are sometimes mentioned, most of the studies and applications described in the literature utilize the porous graphitized carbon (PGC) Hypercarb®.

The properties of PGC come from its highly ordered crystalline structure composed of large flat layers of carbon atoms. It has proven to be unique, behaving as a stronger reversed-phase sorbent than any other existing reversed-phase packing or as a normal-phase sorbent. Separations of both nonpolar and highly polar mixtures can be performed that are impossible with other sorbents. Resolution of anionic and cationic analytes can be achieved in one run. These properties are partly explained by a retention mechanism that is quite different from that of other LC stationary phases. The properties of PGC are discussed here together with some selected applications.

Structure and Characteristics of PGC

PGC is obtained by impregnating a porous silica with a phenol–formaldehyde mixture. This mixture is polymerized within the pores of the silica gel and carbonized at 1000°C. The silica is then removed by dissolution in a concentrated (5 mol L^{-1}) sodium hydroxide solution. Graphitization is performed in the temperature range 2000–2800°C to remove the micropores. The resulting macroporous material has a flat crystalline surface.